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ENZYME-IMMUNOASSAY

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Enzyme-Immunoassay

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Chapter 8

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA): THEORETICAL AND PRACTICAL ASPECTS*

Brian R. Clark and Eva Engvall

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I. INTRODUCTION

Immunoassays are rapidly replacing many other methods used to detect or quantitate substances with important biologic or pharmacologic properties. The high levels of sensitivity and specificity achieved with immunoassays result from the specific, high-affinity, reversible binding of antigens to antibodies, and from the existence of methods for attachment of sensitively detected labels (isotopes, fluorophores, ferritin, free radicals, bacteriophages, and enzymes) to antigens or antibodies. Although isotopes are currently the most extensively used label, the number of sensitive, specific immunoassays employing enzyme tags is expanding rapidly.

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Among the first applications of enzymes as labels was the use of enzyme-antibody conjugates to detect and localize antigenic cellular components by light and electron microscopy.[1] Shortly thereafter, the use of enzyme-antigen and enzyme-antibody conjugates in immunoassays was reported by Engvall and Perlmann,[2] and independently by van Weemen and Schuurs.[3] The many applications and modifications of the enzyme-immunoassay technique that have appeared subsequently attest to its great promise in clinical medicine and research.[4-10]

Enzyme-immunoassays (EIA) are classified into two groups: (1) the heterogeneous EIA, in which the enzyme-labeled antigen or antibody is separated from the enzyme-labeled antigen-antibody complex before measurement of enzyme activity in either fraction, and (2) the homogeneous EIA, in which the enzyme activity of labeled antigen is measured in the presence of labeled antigen-antibody complex, the enzyme moiety of which is sterically inhibited.

The enzyme-linked immunosorbent assay (ELISA), a heterogeneous EIA based on the same principles as the radioimmunoassay (RIA), has been the subject of several reviews.[4,11-13] The only major difference between ELISA and RIA is the use of an enzyme to label the antigen or antibody, rather than a radioactive isotope. Like the RIA, where uncomplexed or free radiolabeled antigen or antibody is separated from radiolabeled antigen-antibody complex (bound radioactivity), the ELISA includes a separation of enzyme-labeled antigen-antibody complex (bound enzyme) from free enzyme-labeled antigen or antibody. The enzymatic activity in the bound or free fraction is quantitated by the enzyme-catalyzed conversion of a relatively nonchromatic or nonfluorescent substrate to a highly chromatic or fluorescent product.

In comparison with RIA, ELISA has several important advantages. In RIA, the most commonly used isotopes (^{125}I and ^{131}I) have short half-lives, and when incorporated into substances, their radioactive disintegrations are destructive to molecular structure. Moreover, the performance of RIA requires special precautions because of the health hazards posed by radioactive isotopes, and the regulations restricting their handling are becoming more stringent. In contrast, enzyme-labeled materials are not hazardous, they have much longer shelf-lives, and the enzyme property of substrate turnover provides an amplification effect in ELISA. However, one limitation of ELISA techniques stems from the relative lack of control of enzyme labeling reactions as compared with radiolabeling procedures. Furthermore, the purification of an enzyme-labeled substance is often difficult or not practical.

An important consideration when comparing RIA and EIA is the greater cost of the instrumentation for quantitation of radioactivity, compared with the cost of equipment required to measure colored or fluorescent solutions. Also, EIA has the potential for complete automation inasmuch as automatic enzyme analysis has already been realized in clinical applications.

II. CLASSIFICATION OF ELISA TECHNIQUES

The following gives a brief description of techniques used in ELISA. The various assays have been classified as either competitive or noncompetitive (immunoenzymometric), depending on whether the technique involves a reaction step in which unlabeled antigen and antigen linked to an enzyme or attached to a solid phase compete for a limited number of antibody binding sites, or whether the antigen or antibody to be measured is allowed to react alone with an excess of immune reactant.

A. Competitive Assays

1. Using Antigen-Enzyme Conjugate

The type of competitive ELISA that utilizes antigen-enzyme conjugates is shown in

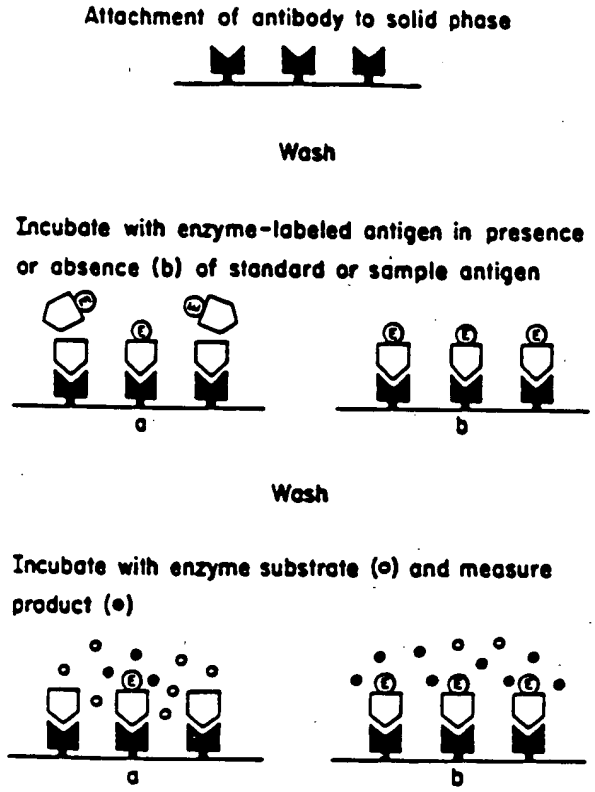

FIGURE 1. Competitive ELISA using antigen-enzyme conjugate and immobilized antibody.

Figure 1. In this scheme, the first operation is the physical or chemical attachment of antibody to a solid phase (step 1). After washing away unattached antibody (step 2), each one of a series of replicate solid-phase units is incubated with a solution containing a fixed concentration of enzyme-labeled antigen and either no unlabeled antigen (step 3b), or a known (but variable) concentration of authentic, standard antigen (step 3a), or an unknown concentration of test antigen from a sample (also step 3a). These reaction mixtures, containing a wetting agent to decrease nonspecific adsorption of enzyme-antigen conjugate to the solid phase, are then incubated at constant temperature until the antigen-antibody reaction attains equilibrium. Following a wash with wetting agent solution (step 4), the set of replicate solid-phase units containing enzyme-labeled antigen-antibody complexes is incubated at constant temperature with a solution containing enzyme substrate (step 5). Subsequently, the enzyme reaction is stopped, and the product concentration is determined using a colorimeter or fluorometer. The measured product concentrations are inversely proportional to the concentrations of standard or test antigen in the incubation solutions of step 3a. This type of competitive ELISA has been applied, for example, to the measurement of rabbit IgG[14] and human chorionic gonadotropin.[15]

2. Using Enzyme-Labeled Antibody

Another type of competitive ELISA employs enzyme-labeled antibody, and the antigen is attached to a solid phase. In this technique (Figure 2), the binding of enzyme-labeled antibody to immobilized antigen (step 3b) is competitively decreased by added

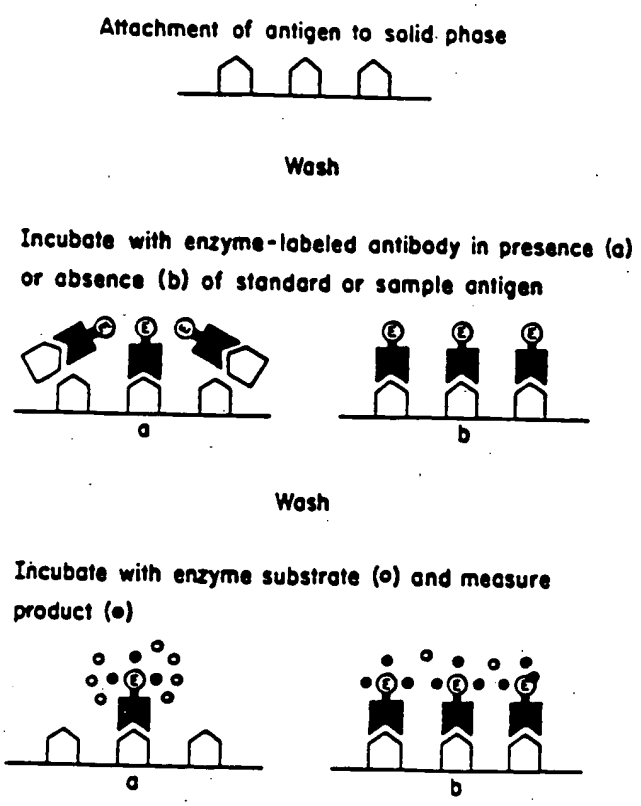

FIGURE 2. Competitive ELISA using antibody-enzyme conjugate and immobilized antigen.

standard or test antigen (step 3a). As in the competitive ELISA with enzyme-labeled antigen, the product concentrations measured in step 5 are inversely proportional to the concentrations of standard or test antigen in the incubation solutions of step 3a. For instance, this type of competitive ELISA has been applied to measure carcinoembryonic antigen.[16]

A variant of the competitive ELISA of Figure 2 employs an enzyme-labeled, secondary antibody, specific for IgG of the animal species in which the primary antibody is elicited. Thus, unlabeled antibody is employed in step 3, and the washed solid phase containing unlabeled antigen-antibody complexes is then incubated with a solution containing enzyme-labeled anti-IgG. After washing, the amount of solid phase-bound enzyme activity is quantitated as above. This technique also has been applied to the quantitation of carcinoembryonic antigen.[16]

B. Noncompetitive Assays

Noncompetitive ELISA techniques are examples of immunoenzymometric assays in which the test antigen is reacted with an excess of antibody, and the extent of the antigen-antibody reaction is measured in a second step. These assays may be classified according to the valency of the antigen. Thus, the single-site, noncompetitive ELISA may employ a univalent antigen, but the two-site or sandwich assay is applicable only to bivalent or polyvalent antigens.

1. Single-Site, Noncompetitive Assay

Two types of single-site, noncompetitive ELISA techniques are, in fact, simple mod-

ifications of the competitive ELISA procedures outlined in Figures 1 and 2. The first type utilizes enzyme-labeled antigen, as in the competitive assay of Figure 1. Instead of incubating labeled and unlabeled antigen together with solid phase-attached antibody (step 3a), standard or test antigen alone is incubated with a moderate excess of immobilized antibody. After washing, excess enzyme-labeled antigen is allowed to bind to unreacted immobilized antibody. The remaining procedure is identical with that of Figure 1, and the enzyme-product concentration (step 5) is inversely proportional to the concentration of standard or test antigen.

The second type of single-site, noncompetitive ELISA employs enzyme-labeled antibody as in the competitive ELISA of Figure 2. Standard or test antigen is incubated separately with a moderate excess of enzyme-labeled antibody. The equilibrium reaction mixture is then added to an excess of immobilized antigen to remove unreacted enzyme-labeled antibody. As in the competitive ELISA of Figure 2, the concentration of enzyme product (step 5) is inversely proportional to the concentration of standard or test antigen. This procedure has been used to measure alpha-fetoprotein.[17]

In both types of single-site ELISA, the second incubation with excess reagent must be of short enough duration that the enzyme-labeled antigen-antibody complex formed in the first incubation does not undergo significant dissociation. The latter condition requires that high-affinity antibody be used so that the antigen-antibody complex has a relatively long half-life. The potential of these assays has not yet been fully realized in enzyme immunoassays. They may prove superior to other ELISA methods with regard to assay sensitivity and protection of the enzyme label from destructive substances present in the test sample.

2. Two-Site or Sandwich Assay

The two-site or sandwich noncompetitive ELISA is shown in Figure 3. Immobilized antibody in excess is incubated with standard or test antigen (step 3). After washing, the immobilized antibody-antigen complex is incubated with an excess of enzyme-labeled antibody which binds to one or more remaining antigenic sites (step 5). Alternatively, the second antibody may be unlabeled, and the procedure is expanded to include an incubation with excess enzyme-labeled third antibody, specific for IgG of the animal species from which the second antibody is elicited. In the latter case, the immobilized and second antibodies must be obtained from different animal species, in order to prevent the binding of enzyme-labeled third antibody directly to the immobilized antibody. In both variants, the concentration of enzyme product (step 7) is directly proportional to the concentration of standard or test antigen (step 3). Examples of the sandwich ELISA include methods to quantitate carcinoembryonic antigen,[18] rat alpha-fetoprotein,[19] and β-aminotransferase.[20]

3. Assays for Measuring Antibody

Another type of noncompetitive ELISA is the indirect method for measuring antibody concentration. This procedure, depicted in Figure 4, employs immobilized antigen and enzyme-labeled second antibody against IgG of the species in which the test antibody has been elicited. This method has been used to measure antibodies to a variety of antigens.[21,22]

C. Factors Involved in the Choice of Assay Design

Although competitive ELISA techniques are specific and easy to execute, they also suffer from several disadvantages. To perform a competitive ELISA of the type illustrated in Figure 1, purified antigen is required for preparation of the enzyme-antigen conjugate. When pure antigen is difficult to prepare, this problem can be avoided by

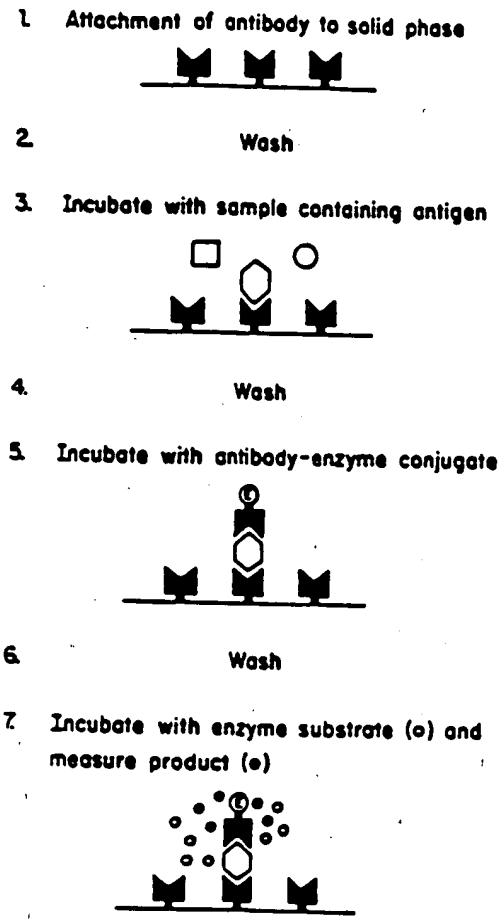

FIGURE 3. Sandwich ELISA for antigens.

use of a variant of the competitive ELISA method diagramed in Figure 2, where an enzyme-labeled second antibody is used to quantitate the amount of first antibody bound to the immobilized antigen.

A far more serious problem in the application of the competitive ELISA, is the incubation of enzyme-labeled antigens or antibodies with test solutions containing serum, urine, or tissue extract. These solutions may contain protein modifying enzymes such as proteases, and noncompetitive enzyme inhibitors, all of which may substantially alter the activity of the enzyme label in the subsequent incubation with enzyme substrate. This problem is avoided in the noncompetitive ELISA techniques where the incubation with test antigen is separate from the incubation with enzyme-labeled antigen or antibody. In addition, the noncompetitive ELISA offers several other advantages. First, since most of these assays employ enzyme-labeled antibodies, the purification and specific enzyme-labeling of individual antigens may be avoided. Thus, the same enzyme-labeling procedure and solid-phase attachment method can be used for different antibodies. Second, the use of a single enzyme-labeled antibody against the IgG fraction of a particular animal species, permits quantitation of all the anitgens used to elicit IgG antibodies in that species. Thus, an enzyme-labeled goat antibody specific for rabbit IgG can be used in the single-site noncompetitive ELISA to measure a variety of antigens for which rabbit IgG antibodies have been obtained. The obvious

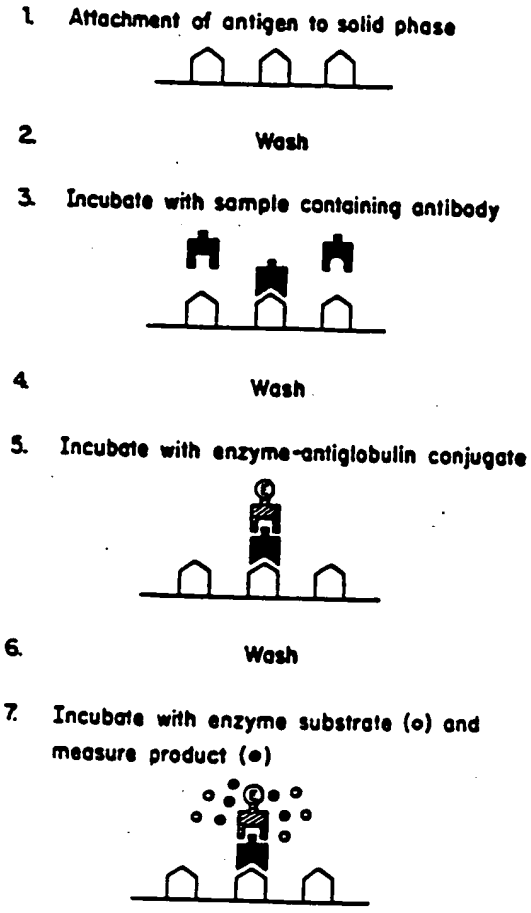

FIGURE 4. Indirect ELISA for antibodies.

advantage of this type of ELISA is that neither antigen nor specific antibody need be conjugated with enzyme. A third advantage of the noncompetitive ELISA is the possibility of binding several enzyme-labeled antibody molecules to a single polyvalent-antigen molecule, thus providing an element of amplification. This may be an advantage in procedures where the ultimate sensitivity has not been attained (i.e., the sensitivity limit set by the affinity between the antigen and antibody). Finally, when sensitivity is not limited by the detection of the enzyme label, the noncompetitive ELISA is inherently more sensitive than the competitive analogue.

III. BASIC METHODOLOGY OF ELISA

A. Selection of Enzyme

The sensitivity of an enzyme immunoassay is directly related to the amplification effect imparted by the enzyme moiety (the formation of many product molecules per test antigen molecule). For a reasonably inexpensive, sensitive, and easily performed ELISA, the enzyme used in the preparation of enzyme-antigen or enzyme-antibody conjugates should meet the following criteria:

1. The enzyme should be relatively stable at 25° and 37°C, and have a shelf-life of at least 6 months at 4°C.
2. The purified enzyme should be commercially available and relatively inexpensive.
3. The activity of the enzyme should be easily measurable using simple colorimetric or fluorimetric methods.
4. Small amounts of enzyme should be detectable. Therefore, the enzyme should have a high substrate turnover number, and the reaction product should have a large coefficient of molar extinction or molar fluorescence.
5. For competitive ELISA techniques, the enzyme should not be affected by biological components of the test sample.

The enzymes that best satisfy these criteria, and are most often employed in ELISA techniques, include alkaline phosphatase from calf intestine, horseradish peroxidase, and *Escherichia coli* β-D-galactosidase. Calf intestine alkaline phosphatase is very stable, and its activity may be quantitated simply and sensitively, using *p*-nitrophenyl phosphate (spectrophotometric detection) or 4-methylumbelliferyl phosphate (fluorometric detection) as a substrate. *p*-Nitrophenyl phosphate is hydrolyzed to *p*-nitrophenol by the enzyme at twice the rate of conversion of 4-methylumbelliferyl phosphate to 4-methylumbelliferone, but the latter is detected at about 10^{-9} *M* compared with about 10^{-6} *M* for *p*-nitrophenol.[18]

E. coli β-galactosidase is also a very stable enzyme, and its activity can be measured using *p*-nitrophenyl-β-D-galactoside or 4-methylumbelliferyl-β-D-galactoside as a substrate. Like calf intestine alkaline phosphatase, this enzyme turns over the former substrate more rapidly, but is, nevertheless, more sensitively detected using the latter.[19]

In many instances, the amount of antigen in the biological test sample is large enough to permit the use of a relatively insensitive colorimetric enzyme-immunoassay. Consequently, use of a *p*-nitrophenyl-derivatized enzyme substrate, combined with colorimetric measurement of the product, *p*-nitrophenol, is usually sufficient. However, in those assays requiring greater sensitivity, use of 4-methylumbelliferyl-derivatized enzyme substrates, combined with fluorimetric quantitation of the product, 4-methylumbelliferone, results, theoretically, in a 100-fold or greater increase in sensitivity. In practice, this has yet to be shown by a direct comparison using the same assay system.

Horseradish peroxidase is also widely used in ELISA techniques, partly because it is less expensive commercially than either of the above two enzymes. However, the determination of its activity involves several sensitive redox reactions, and the substrate, H_2O_2, is unstable.

B. Conjugation of Enzyme to Antigen or Antibody

Procedures for the coupling of enzymes to antigens are more thoroughly discussed elsewhere in this volume. In brief, the conjugation methods used in ELISA techniques employ either a one-step conjugation method where the two components to be covalently attached are mixed together with the cross-linking agent, or a two-step procedure. In the first step of the latter, one of the two components is reacted alone with the coupling agent, and after removal of excess cross-linker, the resultant activated product is added to the second component. In the one-step method, each component cross-links with itself as well as with the other, whereas in the two-step method, only the first component will be self-linked. Glutaraldehyde has been the most frequently used cross-linking agent for coupling enzymes to protein antigens and antibodies. Both one- and two-step coupling procedures have been used.[23]

To eliminate any self-linking of the two components to be coupled, several heterobifunctional cross-linking agents have been devised such that the component to be

activated in the two-step method may react with only one of the two different reactive sites on the coupling agent. Thus, *N*-hydroxysuccinimidyl *m*-maleimidobenzoate (HSMB) was used to form the *m*-maleimidobenzamide derivative of insulin (insulin contains several amino groups, each which may displace the *N*-hydroxysuccimidyl moiety of HSMB, but has no free sulfhydryl groups to react with the maleimido moiety).[24] After removal of excess HSMB, the insulin-*m*-maleimidobenzamide derivative was reacted with *E. coli* β-galactosidase containing free sulfhydryl groups, which reacted with the maleimido moieties of the activated insulin. This two-step method is of general applicability in that any free sulfhydryl groups present in the first component may be blocked with *N*-ethylmaleimide before reaction with HSMB, and if necessary, free sulfhydryl groups may be introduced into the second component by reaction with homocysteine thiolactone. By eliminating self-linking of components, a two-step method using a heterobifunctional coupling agent will result in a higher specific activity of the enzyme conjugate, and thereby increase assay sensitivity.

After formation of enzyme-antigen or enzyme-antibody conjugates, gel filtration may be employed to remove the unlabeled components. This is especially effective for two-stage coupling procedures using heterobifunctional reagents. Another separation method (not yet exploited) is the use of affinity gels containing a covalently linked enzyme inhibitor.

C. Immobilization of Antigen or Antibody

The characteristic that distinguishes ELISA from other EIA is the use of an immune adsorbent to effect a rapid, facile separation of free antigen and antibody from antigen-antibody complex. Antibody and antigen have been covalently attached to cellulose, agarose, and polyacrylamide. Except for magnetic particles,[25] the use of particulate solid phases entails centrifugation in the washing and separation steps. Solid-phase carriers such as beads, discs, and tubes facilitate washing and separation steps. Thus, macromolecular antigens and antibodies have been physically adsorbed to plastic carriers (polystyrene, polyvinyl, polypropylene, polycarbonate), and to silicone rubber, or treated glass. Indeed, part of the success of ELISA methods arise from the use of disposable polystyrene microtiter plates or tubes as the solid-phase carriers.

Most proteins adsorb to plastic surfaces, probably as a result of hydrophobic interactions between nonpolar protein substructures and the nonpolar plastic matrix. The rate and extent of coating will depend on the diffusion coefficient of the adsorbing molecule, the ratio of the surface area to be coated to the volume of coating solution, the concentration of the adsorbing substance, the temperature, and the duration of the adsorption reaction. Polystyrene has been the most widely used support in ELISA methods because it can be coated easily and reproducibly. There are however, several disadvantages to using polystyrene or any other plastic as solid phase. One difficulty arises because the antigen or antibody is not covalently bound, but only physically adsorbed. This type of immunoadsorbent bleeds (i.e., loses adsorbed protein during washes and incubations) and adsorbed antibodies also undergo denaturation with loss of antigen-binding activity.[8] The loss of adsorbed antigen or antibody (whether by desorption or denaturation) lowers the precision of the assay, and probably also affects its sensitivity, especially in competitive ELISA techniques. Moreover, the extent of denaturation of the adsorbed antigen or antibody may result in a loss of binding capacity such that the specificity of the assay is decreased, because the nonspecific binding of labeled components contributes significantly to the total bound enzyme activity. Another disadvantage is that plastic surfaces have a limited capacity of adsorption. However, the ease and rapidity of separation of antigen-antibody complex from free antigen and antibody often compensates for these drawbacks.

The adsorption process, unlike antigen-antibody interactions, is nonspecific. Thus, during the incubation of the immobilized antigen or antibody with enzyme-labeled antigen or antibody, the latter binds specifically to the immobilized immune component, but also may be adsorbed directly onto the solid phase. This nonspecific adsorption of enzyme activity can be minimized by inclusion of a neutral detergent (such as Triton® X-100 or Tween® 20) that does not interfere significantly with the antigen-antibody reaction. Nonionic detergents can thus be added in concentrations that prevent formation of new hydrophobic interactions between added proteins and the solid phase, but that also do not appreciably disrupt hydrophobic bonds already formed between the previously adsorbed antigen or antibody and the plastic surface.

Besides nonspecific adsorption of enzyme label to the solid phase, another important factor affecting specificity is the completeness of separation of the adsorbed enzyme-labeled antigen-antibody complex from solution containing free enzyme-labeled antigen or antibody. Most plastics display a negative electric potential at their surfaces (the so-called zeta potential). This may be modified, but not eliminated by adsorption of protein.[26] The residual electrostatic field at the solid-solution boundary gives rise to a diffuse double-layer of ions at the interface. Depending on the plastic surface architecture, this double layer, containing free enzyme-labeled antigen or antibody, may be difficult to displace except by vigorous or prolonged washing. Variation in the extent of removal of nonadsorbed enzyme label present in the diffuse double-layer also may be a factor affecting the precision of the assay.

IV. PERFORMANCE CHARACTERISTICS OF ELISA METHODS

A. Sensitivity

Two major factors limit the sensitivity of both RIA and the ELISA: the binding affinity between antigen and antibody, and the level of detection of the radioisotope or enzyme employed as label. High-affinity antibodies display affinity constants on the order of 10^{10} $(M/l)^{-1}$ to 10^{12} $(M/l)^{-1}$, resulting in sensitivities of about 10^{-10} to 10^{-12} M or from 20 down to 0.2 fmol of antigen per 0.2 ml reaction volume. Methods for detecting radioisotopes or enzymes can detect 0.02 fmol in 0.2 ml. Hence, the affinity between antigen and antibody probably limits the sensitivity in those immunoassay procedures that use the most sensitive label-detection methods.

When RIA and ELISA methods for the same antigen and antibody combination are compared, any loss of sensitivity in the ELISA may be ascribed to a decrease in the antigen-antibody binding affinity. The decreased affinity may result from structural changes accompanying the coupling of enzyme to antigen or antibody, and/or it may be caused by molecular distortion in the immobilized antigen or antibody. However, despite the possible occurrence of these effects, direct comparisons of EIA and RIA have revealed that the two methods have nearly the same sensitivities.[27-30]

Among the various ELISA techniques, the most sensitive is, in theory, the two-site or sandwich ELISA. Any enhancement of sensitivity achieved with this noncompetitive assay (compared with competitive ELISA techniques) most probably results from the use of excess reagents in each step of the procedure. This ensures a maximum extent of reaction between antigen and antibody, thereby permitting measurement of lower antigen concentrations.

Similarly, one-site noncompetitive ELISA methods also should be, (theoretically), more sensitive than competitive ELISA techniques. In fact, our data (to be published) and those of others[17] show that the one-site ELISA can be made more sensitive than the corresponding competitive method. However, the difference in sensitivity is not large, and the longer procedure of the noncompetitive ELISA may not be compensated by the relatively small gain in sensitivity. The greater value of the one-site ELISA lies

in the separate incubation of the enzyme-labeled component subsequent to the incubation step with solution containing the test antigen, and other substances present in the biological test sample.

B. Specificity and Accuracy

The principal determinant of specificity in immunoassays (in general) and ELISA techniques (in particular) is the antibody. Specificity of antibody preparations can be enhanced by using immunosorbents to remove nonspecific or interfering antibodies. Even then, an antibody may cross-react with antigens similar in structure to the immunizing antigen, and this limits the specificity. Finally, an equally critical factor is the purity of the antigen used as immunogen and assay standard.

One major cause of decreased specificity in ELISA methods is the nonspecific absorption of enzyme-labeled antigen or antibody to the solid phase. As discussed previously, inclusion of a nonionic detergent in the incubation solutions almost always substantially reduces this effect.

A serious decrease in specificity may occur in the two-site or sandwich ELISA as a result of the presence of rheumatoid factor in the test sample.[17,31] This substance (when present) binds enzyme-labeled secondary antibody to the immobilized primary antibody, thus falsely elevating the apparent amount of enzyme-labeled antigen-antibody complex. Since the binding involves the F_c segment of the antibody molecule, this nonspecific effect can be eliminated by employing enzyme-labeled Fab or Fab' antibody fragments in place of the enzyme-labeled secondary antibody.

Finally, erroneous results may be obtained in competitive ELISA techniques as a result of destruction or alteration of the enzyme label during the incubation with biological test sample, for reasons discussed earlier in this chapter. This problem does not arise in noncompetitive ELISA methods. However, both types of ELISA may be subject to error introduced by variable, test sample-dependent bleed of the solid-phase adsorbent.

The accuracy of an ELISA can be assessed by measuring the recovery of added, authentic antigen from test solutions, and by correlating assay results with those obtained using RIA or other methods. Where comparisons have been made between EIA and RIA, correlation coefficients greater than 0.9 were nearly always obtained.[17,32-36]

C. Precision and Reproducibility

ELISA and RIA have not been compared extensively regarding precision and reproducibility. In one study, the interassay variation was 10% for EIA, compared with 8% for RIA.[17] The same study reported an interassay variation of 7% for a sandwich assay. With regard to precision, intraassay variation usually lies in the RIA range, i.e., 5 to 15%,[27,32-38] despite the view that determination of enzyme activity is inherently more imprecise than measurement of radioactivity.

One source of intraassay variability in ELISA methods may be nonuniform adsorption of antigen or antibody onto plastic surfaces during the immobilization step. This may be caused by inhomogeneities in the plastic material, or may arise from nonuniform-charge effects over the plastic surface. These sources of variability are difficult to control. ELISA microtiter plates with high homogeneity are available commercially, and surface-charge effects can be reduced by using a static charge eliminator, or by keeping the plastic in contact with a damp towel.

D. Protocol

The time required to complete an ELISA procedure will vary with the type of ELISA technique, but is usually from one to several days. As with RIA, many samples can

be processed concurrently (a microtiter plate contains 96 wells, and several such plates can be processed simultaneously).

No unusual apparatus is required for ELISA methods. Automatic pipettes with a row of up to eight delivery tips are useful for additions to microtiter plates, and these are available commercially. A constant temperature incubator is useful, but not a necessity. Instrumentation is simple. A colorimeter, spectrophotometer, or fluorometer is all that is required.

The processing of ELISA data has not been described extensively. We have found that the data may be transformed as in RIA techniques, i.e., the logit-log transformation, permiting linear-dose interpolation. More details of the processing of EIA data are presented elsewhere in this volume.

Finally, EIA in general, and ELISA in particular, are well suited to automation. A modular apparatus containing pipetting and dilution stations is already in use with RIA, and can be used for EIA and ELISA. Such apparatus can also contain a colorimeter or spectrophotometer. Mechanized systems for performing ELISA assays have been described.[36] Such systems will undoubtedly undergo rapid development as the application of ELISA techniques continues to expand.

V. AREAS OF FUTURE DEVELOPMENT

The application of ELISA techniques to the quantitation of drugs and nonprotein biological substances has not enjoyed the rapid and extensive development as have the assays for macromolecules. This may be due (in part) to the difficulty in preparing enzyme conjugates of small molecules in a way that preserves the antigen-antibody binding affinity. Future developments will undoubtedly include improvements in methods for the controlled and specific linking of enzymes and antigens, and for purification of the resultant conjugates.

Another area where development is needed is the covalent bonding of antigens and antibodies to activated plastic surfaces as a means of eliminating the undesirable aspects of physical adsorption techniques.

Finally, further development of automated procedures will hasten the replacement of the RIA procedures used in clinical medicine. Thus, there are ample reasons to expect that the EIA in general and the ELISA in particular will in the near future enjoy the present eminence of the RIA.

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